CHAPTER FIVE

Progress Towards RNAi-Mediated Insect Pest Management

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Contents

Abstract

Gene suppression via RNA interference (RNAi) provides an alternative strategy for insect pest management. The ingestion by insects of double-stranded RNAs targeting essential insect genes can trigger RNAi and lead to growth inhibition, developmental aberrations, reduced fecundity, and mortality. This RNAi response is particularly acute in certain coleopteran species, most notably the western corn rootworm, a devastating pest impacting corn production in the United States. The development of next-generation rootworm-protected corn hybrids includes an RNAi-based trait that provides a mode of action distinct from those of *Bacillus thuringiensis* insecticidal protein-based traits currently used for rootworm pest management. Unfortunately, many insect species including important lepidoptera and hemiptera pests appear largely recalcitrant in their response to environmental RNA, suggesting biological barriers that thus far limit the utility of RNAi for agricultural pest management. This review will highlight recent

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efforts to understand the barriers to RNA delivery in recalcitrant insect species, describe recent advances in the commercial development of insect-protected crops and biological insecticides utilizing RNAi, and discuss this strategy in the context of an integrated pest management approach.

1. INTRODUCTION

Eukaryotic cells possess a conserved pathway by which exogenously applied and endogenously expressed double-stranded (ds) RNAs direct the degradation of complementary endogenous messenger RNA (mRNA) transcripts within a cell resulting in sequence-specific gene suppression. This phenomenon is referred to as RNA interference (RNAi) (Fire et al., 1998; Hannon, 2002). In plants and animals, RNAi provides one line of defence against RNA viruses and foreign dsRNA molecules. Small endogenous RNAs known as micro RNAs are also processed by a related pathway to regulate tissue-specific patterns of gene expression primarily via translational regulation (Bartel, 2009). Long non-coding RNAs also play a prominent role in the epigenetic regulation of gene expression (Lee, 2012). It is now clear that far more of the genome is transcribed than previously thought (Djebali et al., 2012) and that RNA, in addition to being the obligate messenger and facilitator of protein synthesis in the cell, is also a central player in the regulation of eukaryotic gene expression.

The general mechanism of dsRNA-mediated degradation of mRNA transcripts is understood (Tomari and Zamore, 2005). Long dsRNAs are a substrate for RNAse III-like proteins referred to as Dicer or Dicer-like proteins. Dicer appears to preferentially initiate dsRNA cleavage at the ends of the dsRNA, making successive cleavages to generate 21- to 24-bp silencing (si) RNA duplexes (Elbashir et al., 2001). The resulting siRNA duplexes are loaded into a multiprotein complex called the RNA-induced silencing complex (RISC) where the passenger (sense) strand is removed and the guide (antisense) strand remains to target mRNA for silencing. The guide strand in the RISC enables Watson–Crick base pairing of the complex to complementary mRNA transcripts and enzymatic cleavage of the target mRNA by a class of proteins referred to as Argonaute proteins, thereby preventing mRNA translation. Accordingly, this mechanism of gene suppression is highly sequence specific. The ability to selectively down-regulate genes via RNAi has proved to be valuable, particularly in insects for which genetic tools are not readily available to study gene function (Belles, 2010).

The surprising observation that ingested dsRNAs can trigger gene suppression in the nematode, *Caenorhabditis elegans*, (Timmons and Fire, 1998; Timmons et al., 2001) offered hope that the oral delivery of dsRNA could modulate gene expression in other invertebrates, including insects, for the purpose of pest management.

The ability of herbivorous insects to adapt to insecticide use in agricultural systems presents an ongoing challenge for pest management. Over 20 years ago, more than 500 species of arthropods were documented with resistance to one or more pesticides (Georghiou et al., 1991); an updated dataset can be found at http://www.pesticideresistance.com/index.php. The development of insecticides with new modes of action (MOAs) is a priority, but so is the implementation of resistance management strategies to prolong the use of existing insecticides for use in agriculture and public health (http://www.irac-online.org/). Insect-protected crops, expressing insecticidal proteins derived from the bacterium, *Bacillus thuringiensis* (Bt), represent a significant fraction of the >170 M ha of transgenic crops cultivated worldwide (James, 2013) and provide excellent control of many economically important insect pest species. Consequentially, such crops also impose strong selective pressure on insects to adapt. As is the case for synthetic- and biological insecticides, alternative MOAs for insect-protected crops are needed, either because some insect species are not sensitive to Bt insecticidal proteins or because some have evolved field resistance to efficacious Bt proteins (Storer et al., 2010). To that end, RNAi-mediated insect control represents a significant opportunity.

In 2007, papers from Baum et al. and Mao et al. demonstrated that transgenic plants expressing insect-derived dsRNAs could impact the growth and development of insect herbivores. Corn plants expressing a dsRNA hairpin that targets the vacuolar ATPase A subunit gene in western corn rootworm (WCR), *Diabrotica virgifera* virgifera, caused severe rootworm stunting and exhibited significant protection from rootworm feeding damage, consistent with artificial diet feeding assays demonstrating the insecticidal activity of such dsRNA species (Baum et al., 2007). *Arabidopsis* plants expressing a dsRNA hairpin that targets a cytochrome P450 monooxygenase gene in the cotton bollworm, *Helicoverpa armigera*, led to decreased bollworm tolerance to the cotton sesquiterpene aldehyde, gossypol (Mao et al., 2007), consistent with the proposed role of this enzyme in gossypol detoxification. The former example has advanced towards commercial development as a component of next-generation rootworm-protected corn hybrids (Kupferschmidt, 2013) and will be discussed further in this review.

Numerous studies have since explored the sensitivity of diverse insect species to ingested dsRNAs. In addition, several useful reviews have been published on the subject of RNAi and insects to which we refer the reader (e.g. Aronstein et al., 2011; Belles, 2010; Burand and Hunter, 2013; Gu and Knipple, 2013; Huvenne and Smagghe, 2010; Li et al., 2013b; Terenius et al., 2011; Yu et al., 2013; Zhang et al., 2012a, 2013a). Rather than recapitulate these publications, this review will focus on the development and use of RNAi strategies for insect control in agriculture, highlight efforts to understand the barriers to RNA delivery in recalcitrant insect species, describe recent advances in the commercial development of insect-protected crops and biological insecticides utilizing RNAi, and discuss this strategy in the context of an integrated pest management (IPM) approach.

2. ENVIRONMENTAL RNAi

Definitions have been proposed to discuss the various aspects of RNAi in plants and animals (Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008). Cell-autonomous RNAi refers to the RNAi response that individual cells carry out when encountering dsRNA, a response that is executed by a broadly conserved or core RNAi machinery found in eukaryotic cells. Non-cell autonomous RNAi includes the phenomenon of systemic RNAi—the movement of a silencing signal, presumably siRNA and/or dsRNA, from cell to cell and from one part of an organism to another. Non-cell autonomous RNAi also includes the phenomenon of environmental RNAi which, as its name suggests, refers to the triggering of RNAi by environmental exposure to dsRNA by soaking or feeding. Environmental RNAi may or may not be followed by systemic movement of the silencing signal, perhaps a key step in determining the biological activity of a dsRNA.

Components of the core RNAi machinery are readily identified in insect species whose genomes have been sequenced (e.g. Honeybee Genome Sequencing Consortium, 2006; International Silkworm Genome Consortium, 2008; The International Aphid Genomics Consortium, 2010; Tomoyasu et al., 2008; Tribolium Genome Sequence Consortium, 2008) and evidence for functional RNAi reported in a wide range of insect species encompassing the orders Coleoptera, Diptera, Dictyoptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Neuroptera, and Orthoptera. In order to be useful as an insect control agent in agriculture, environmental RNAi must first be operational: dsRNA must be delivered to the insect either by ingestion or by penetration of the insect cuticle in order

to trigger an RNAi response in recipient cells. For transgenic plant applications and for most topical spray applications, delivery via ingestion is likely the dominant route of entry. An overall picture of the responsiveness of different insect species and orders to ingested dsRNAs is emerging (for reviews, see Huvenne and Smagghe, 2010; Li et al., 2013b; Terenius et al., 2011). In feeding studies, dsRNAs are supplied by droplet feeding, incorporated into an artificial diet, or applied to leaf/plant tissue suitable for insect feeding. While the dose of dsRNA consumed is not known in many instances, this variable is less important than the concentration of dietary dsRNA presented to the insect: the effective concentration of a dsRNA active ultimately determines its utility for insect control. In reviewing these studies, we determined or estimated, whenever possible, the dietary concentration of dsRNA tested in parts per million (ppm) or parts per billion (ppb) in order to normalize the results and compare among studies. A summary of published studies evaluating the effects of ingested dsRNAs or siRNAs on insect species is presented in Table 5.1.

3. INSECT SENSITIVITY TO ENVIRONMENTAL RNAi

3.1. Coleoptera

A number of coleopteran species, including the WCR (*D. virgifera* virgifera), southern corn rootworm, *Diabrotica undecimpunctata* howardi, Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, and canola flea beetle, *Phyllotreta striolata*, are remarkably sensitive to ingested dsRNAs with LC_{50} values in the range of 1–10 ppb (Table 5.1, Baum et al., 2007; Bolognesi et al., 2012; Zhao et al., 2008). This sensitivity to environmental RNAi extends to both the larval and adult stages (Rangasamy and Siegfried, 2012; Zhao et al., 2008). To our knowledge, no insect species outside the Order Coleoptera approaches this level of sensitivity to ingested dsRNA, with most studies of successful oral delivery in other insect orders reporting LC_{50} values >10 ppm. This potent environmental RNAi response is not necessarily shared among all coleopteran species, however, as studies with the red flour beetle, *Tribolium castaneum*, and cotton boll weevil, *Anthonomus grandis*, suggest (Baum et al., 2007; Whyard et al., 2009).

Some aspects of the RNAi response in corn rootworm are instructive. Of the 290 gene targets screened by Baum et al. (2007), approximately 2/5 caused rootworm mortality or stunting in surface overlay diet bioassays when applied at the relatively low concentration of $\sim$50 ng/cm^2, or roughly equivalent to 0.1 ppm in diet (see Baum et al., 2011 for a listing of efficacious

Table 5.1 Sensitivity of insect species to ingested dsRNAs

Organism	Target gene product	Stage	Assay	Mortality or stunting	LC_{50} or (concentration tested)	mRNA silencing	Reference
Coleoptera							
Diabrotica virgifera virgifera	Multiple targets	Neonates	Artificial diet	Yes	1–10 ppb[a]	Yes	Baum et al. (2007)
	V-ATPase A	Neonates	Transgenic plant	Yes	ND	Yes	Baum et al. (2007)
	Snf7	Neonates	Artificial diet	Yes	4.3 ppb	Yes	Bolognesi et al. (2012)
	Snf7	Neonates	Transgenic plant	Yes	ND	Yes	Bolognesi et al. (2012)
Diabrotica undecimpuctata howardii	Snf7	Neonates	Artificial diet	Yes	1.2 ppb	Yes	Bolognesi et al. (2012)
	V- ATPase A and E	Neonates	Artificial diet	Yes	(~0.1 ppm)[a]	Yes	Baum et al. (2007)
	α-Tubulin	Neonates	Artificial diet	Yes	(~0.1 ppm)[a]	Yes	Baum et al. (2007)
Leptinotarsa decemlineata	V-ATPase A and E	Neonates	Artificial diet	Yes	~10 ppb[a]	–	Baum et al. (2007)
	Multiple targets	Neonates	Leaf tissue	Yes	ND	Yes	Zhu et al. (2011)
Phyllotreta striolata	Arginine kinase	Adults	Leaf tissue	Yes	0.8 ppb	Yes	Zhao et al. (2008)
Tribolium castaneum	V-ATPase E	Neonates	Flour	Yes	2.5 ppm	Yes	Whyard et al. (2009)

Diptera							
Aedes aegypti	V-ATPase A	Adults	Artificial diet	–	(1000 ppm)	Yes	Coy et al. (2012)
	Multiple targets	First instars	Water	Yes	(200, 500 ppm)	Yes	Singh et al. (2013)
	ATP-dependent efflux pump	Second instars	Water	–	30 ppm	Yes	Figueira-Mansur et al. (2013)
Anopholes gambiae	Chitin synthase 2	Third instars	Artificial diet	Yes	–	Yes	Zhang et al. (2010)
Anopheles stephensi	3-HKT	First instars	*Chlamydomonas*	Yes	ND	Yes	Kumar et al. (2013)
Bactrocera dorsalis	Multiple targets	Adults	Artificial diet	Yes/No	(2000 ppm)	Yes/No	Li et al. (2011b)
Glossina morsitans morsitans	Tsetse EP	Male adults	Blood meal	No	(>400 ppm)	Yes	Walshe et al. (2009)
	Transferrin	Male adults	Blood meal	No	(>400 ppm)	No	Walshe et al. (2009)
Hemiptera							
Acyrthosiphon pisum	Aquaporin	Six-day-old nymphs	Artificial diet	No	(1000–5000 ppm)	Yes	Shakesby et al. (2009)
	V-ATPase E	First instars	Artificial diet	Yes	3.4 ppm	Yes	Whyard et al. (2009)

Continued

Table 5.1 Sensitivity of insect species to ingested dsRNAs—cont'd

Organism	Target gene product	Stage	Assay	Mortality or stunting	LC_{50} or (concentration tested)	mRNA silencing	Reference
	V-ATPase E	Neonates	Artificial diet	No	ND	No	Christiaens et al. (2014)
	Hunchback (hb)	Neonates	Artificial diet	Yes	(750 ppm)	Yes	Mao and Zeng (2012)
Bactericerca cockerelli	Multiple targets	Adults	Artificial diet	Yes	(500–1000 ppm)	Yes	Wuriyanghan et al. (2011)
Bemisia tabaci	V-ATPase subunit A, rpL19	Adults	Artificial diet	Yes	3,11 ppm	Yes	Upadhyay et al. (2011)
Lygus lineolaris	Inhibitor of apotosis	Neonates	Artificial diet	No	(1000 ppm)	No	Allen and Walker (2012)
Myzus persicae	RACK-1, COO2	Nymphs	Transgenic plant	Yes	ND	Yes	Pitino et al. (2011)
	Hunchback (hb)	Neonates	Transgenic plant	Yes	ND	Yes	Mao and Zeng (2014)
Nilaparvata lugens	Trehalose PO4 synthase	Third instars	Artificial diet	?	(500 ppm)	Yes	Chen et al. (2010)
	V-ATPase E	2nd instars	Artificial diet	No	(50 ppm)	Yes	Li et al. (2011a)
	Multiple targets	Neonates	Transgenic plant	No	ND	Yes	Zha et al. (2011)

Peregrinus maidis	V-ATPase B and D	Third instars	Artificial diet	Yes	(500 ppm)	Yes	Yao et al. (2013)
Rhodnius prolixus	Nitroporin 2	Second instars	Artificial diet	NA	(1000 ppm)	Yes	Araujo et al. (2006)
Sitobion avenae	Multiple targets	Third instars	Artificial diet	Yes	(3 – 7.5 ppm)	Yes	Zhang et al. (2013b)
Hymenoptera							
Apis mellifera	Vitellogenin	Second instars	Natural diet	Nonspecific	(500–3000 ppm)	Yes	Nunes and Simões (2009)
Solenopsis invicta	PBAN/pyrokinin	Fourth instars	Artificial diet	Yes	(1000 ppm)	ND	Vander Meer and Choi (2013)
	GNBP	Workers	Artificial diet	Yes	(200 ppm)	ND	Zhao and Chen (2013)
Isoptera							
Reticulitermes flavipes	Cellulase	Workers	Paper discs	Yes	(5.1 μg/cm^2)	Yes	Zhou et al. (2008)
	Hexamerin	Workers	Paper discs	Yes	(2.2 μg/cm^2)	Yes	Zhou et al. (2008)
Lepidoptera							
Chilo infuscatellus	CiHR3 moulting factor	Third instars	Corn kernels	Yes	(250 ppm)	Yes	Zhang et al. (2012c)
Epiphyas postvittana	Carboxylesterase	Third instars	Droplet	No	(4000 ppm)	Yes	Turner et al. (2006)
	Pheromone bp	Third instars	Droplet	No	(4000 ppm)	Yes	Turner et al. (2006)

Continued

Table 5.1 Sensitivity of insect species to ingested dsRNAs—cont'd

Organism	Target gene product	Stage	Assay	Mortality or stunting	LC_{50} or (concentration tested)	mRNA silencing	Reference
Helicoverpa armigera	CYP6AE14	Third instars	Transgenic plant	Yes	ND	Yes	Mao et al. (2007)
	GST	Third instars	Transgenic plant	No	ND	Yes	Mao et al. (2007)
	CYP6AE14	Third instars	Transgenic plant	Yes	ND	Yes	Mao et al. (2011, 2013)
	AchE receptor	Neonates	Artificial diet	Yes	(~0.35 ppm)	Yes	Kumar et al. (2009)
	AchE receptor	Neonates	Leaf tissue	Yes	(~0.35 ppm)	–	Kumar et al. (2009)
	Ecdysone receptor EcR	Second instars	Transgenic plant	Yes	ND	Yes	Zhu et al. (2012)
	Ecdysone receptor EcR	Third instars	Artificial diet (Ec)[b]	Yes	ND	Yes	Zhu et al. (2012)
	HaHR3 moulting factor	Third instars	Transgenic plant	Yes	ND	Yes	Xiong et al. (2013)
	HaHR3 moulting factor	Third instars	Artificial diet (Ec)	Yes	ND	Yes	Xiong et al. (2013)

	CYP6B6	Third instars	Artificial diet (Ec)	Yes	ND	Yes	Zhang et al. (2013c)
	Ultraspiracle protein, EcR	Third instars	Artificial diet	Yes	(1000 ppm)	Yes	Yang and Han (2014)
Manduca sexta	V-ATPase E	Neonates	Artificial diet	Yes	11 ppm	Yes	Whyard et al. (2009)
Ostrinia nubilalis	Chitinase	Neonates	Artificial diet	Yes	(2500 ppm)	Yes	Khajuria et al. (2010)
Plutella xylostella	CYP6BG1	Fourth instars	Droplet		(~800 ppm)	Yes	Bautista et al. (2009)
	Rieske protein	Second instars	Leaf tissue	Yes	(6 μg/cm^2)	Yes	Gong et al. (2011)
	AchE receptor	Second instars	Leaf tissue	Yes	53.7 ppm	-	Gong et al. (2013)
Sesamia nonagriodes	JH esterase JHER	First to sixth instars	Artificial diet (Ec)	No	ND	Yes	Kontogiannatos et al. (2013)
Spodoptera exigua	Chitin synthase A	Neonates	Artificial diet (Ec)	Yes	ND	Yes	Tian et al. (2009)
	β1 integrin subunit	Fourth instars	Leaf tissue	Yes	100–200 ppm	–	Surakasi et al. (2011)
	Ecdysone receptor EcR	Second instars	Transgenic plant	Yes	ND	Yes	Zhu et al. (2012)

Continued

Table 5.1 Sensitivity of insect species to ingested dsRNAs—cont'd

Organism	Target gene product	Stage	Assay	Mortality or stunting	LC_{50} or (concentration tested)	mRNA silencing	Reference
Spodoptera litura	Aminopeptidase N	Neonates	Artificial diet	No	ND	No	Rajagopal et al. (2002)
Spodoptera frugiperda	Allatostatin C	Fifth instars	Droplet	NA	(600 ppm)	Yes	Griebler et al. (2008)
	Allototropin 2	Fifth instars	Droplet	NA	(600 ppm)	Yes	Griebler et al. (2008)
	SfT6 serine protease	Fourth instars	Droplet	NA	(600 ppm)	Yes	Rodríguez-Cabrera et al. (2010)
Orthoptera							
Gryllus bimaculatus	Sulfakinins	Adults	Droplet	NA	(100–600 ppm)	–	Meyering-Vos and Müller (2007)
Locusta migratoria	Multiple targets	Fourth instars	Artificial diet	No	(~240 ppm)	No	Luo et al. (2013)
Schistocerca gregaria	Tubulin, GAPDH	Adults	Artificial diet	No	ND	No	Wynant et al. (2012)

[a]Estimated from sample overlay assays in which 20 μL samples are infused into 200 μL artificial diet.
[b]Ec = dsRNA expressed in *E. coli*.
ND, not determined or reported; NA, not applicable.

targets). While the suppression of certain gene transcripts did not result in a phenotypic response (Baum et al., 2007, supplemental figure 3), it is clear that the number of specific gene targets available for successful environmental RNAi is large. Sectioning of the V-ATPase A coding region into six ~300 bp dsRNAs did not reveal dramatic differences in efficacy, suggesting that a single dsRNA of this size provides a reasonable sampling of target knockdown efficacy and phenotype. One of the targets identified by Baum et al. (2007), a *Snf*7 ortholog, was selected for a more detailed study of the RNAi response (Bolognesi et al., 2012). *Snf*7 dsRNAs shorter than 50 bp exhibited dramatically reduced activity in the corn rootworm feeding assay. This study employed a single *Snf*7 27-mer sequence embedded in neutral sequences of varying length, ruling out the possibility that differences in siRNA composition accounted for differences in activity. An *in situ* study of RNA uptake further demonstrated that a Cy3-labelled 240 bp *Snf*7 dsRNA was taken up by rootworm midgut epithelial cells while a Cy3-labelled 21 bp *Snf*7 siRNA was not, corroborating the size dependency of the RNAi response observed in feeding assays (Bolognesi et al., 2012). Likewise, injection studies with corn rootworm larvae have demonstrated the inability of siRNAs to produce an RNAi response leading to rootworm mortality (Khajuria et al., 2013). Finally, the RNAi response in corn rootworm appears to be systemic as judged by qPCR analysis of *Snf*7 mRNA transcripts in isolated midgut and cadaver tissues (Bolognesi et al., 2012).

Elements of the rootworm RNAi response can be found in another coleopteran species, the red flour beetle *T. castaneum*, which has become a model system for studying systemic RNAi in insects (Miller et al., 2012). Injection studies with a transgenic *gfp-Tribolium* line permitted visualization of silencing as suppression of green fluorescent protein (gfp) fluorescence. The RNAi response was observed to be dose-dependent, systemic, and likewise dependent on the size of the dsRNA. Injection of ~60 ng of a 520-bp *gfp* dsRNA/last instar larva resulted in detectable silencing of *gfp*, demonstrating that this coleopteran species is very sensitive to systemic RNAi. The efficiency of systemic RNAi appears to drop off with shorter dsRNA molecules although the precise breakpoint was not determined. Adopting a strategy similar to that used for characterization of the corn rootworm *Snf*7 dsRNA (Bolognesi et al., 2012), a 30-bp *gfp* dsRNA fused to a 30-bp neutral *Ultrabithorax* (Ubx) dsRNA was shown to be more effective than the 30-bp *gfp* dsRNA alone in triggering a systemic RNAi response, presumably due to less efficient cellular uptake of the smaller dsRNA. Comparing a series of

siRNAs and larger dsRNAs targeting the *Tc-achaete-scute-homolog* (*Tc-ASH*) and *Ultrabithorax* (*UBX*) genes in *Tribolium*, Wang et al. (2013) reported that dsRNAs provided greater silencing for longer periods of time resulting in developmental phenotypes. Interestingly, the silencing observed with injected siRNAs (0.4–0.5 μg) was short-lived, lasting from days 2 to 4, and did not result in a phenotypic response. The dose-dependent systemic RNAi response observed in *Tribolium* is consistent with the concentration-dependent environmental RNAi response observed in *Diabrotica* that permitted calculation of LC_{50} values (Baum et al., 2007). The steepness of those concentration–response curves may reflect a threshold effect, as has been suggested for *Tribolium* (Miller et al., 2012). Like other insect species, neither *Tribolium* nor *Diabrotica* contains a recognizable RNA-dependent RNA polymerase that could amplify the production of dsRNAs for a systemic response (Baum et al., 2007; Tomoyasu et al., 2008). Despite the apparent absence of this amplification mechanism, corn rootworm larvae are remarkably sensitive to ingested dsRNAs and require no more than a 3-h exposure to dietary dsRNA to observe a lethal phenotype (Bolognesi et al., 2012). Comparative genomic analysis of the *Tribolium* and *Diabrotica* genomes may shed light on the apparent differences in sensitivity to ingested dsRNAs observed between these two species.

3.2. Diptera

Belles (2010) provides some historical context for the pioneering RNAi studies conducted with the fruit fly, *Drosophila melanogaster*, a model system for understanding both the mechanism of RNAi and its role in mediating antiviral immunity in invertebrates (Nayak et al., 2013). Reverse genetic studies of gene function have been enabled through the use of cultured cells, employing both genome-wide (Boutros and Ahringer, 2008; Boutros et al., 2004) and pathway-specific screens (e.g. Clemens et al., 2000). Functional studies of genes involved in *Drosophila* development have relied on microinjection of dsRNA into embryonic tissue (e.g. Kennerdell and Carthew, 1998; Koizumi et al., 2007; Pilot et al., 2006). Libraries of transgenic *Drosophila* lines containing conditionally expressed dsRNA hairpins have been generated for use in whole-animal RNAi screens (e.g. Dietzl et al., 2007). Abundant tools for both *in vivo* and *in vitro* RNAi studies in *Drosophila*, as well as relevant literature, can be found at http://www.flyrnai.org/.

Drosophila appears deficient in systemic RNAi when confronted with endogenously expressed dsRNA hairpins (Roignant et al, 2003), but is

capable of mounting a systemic RNAi response to viral infection (Saleh et al., 2009). The systemic RNAi response to injected dsRNAs is also limited. Using transgenic *Drosophila* lines expressing Gal4-regulated enhanced green fluorescent protein (EGFP) or GFP, Miller et al. (2008) evaluated the systemic RNAi response in last instar larvae and reported that only hemocytes were responsive to injected (hemolymph) dsRNA, whereas in *Tribolium* larvae, virtually all cell types were responsive. Co-expression of an EGFP dsRNA hairpin resulted in down-regulation of EGFP in all tissues examined, demonstrating that the limited systemic RNAi response observed in *Drosophila* larvae is not due to the absence of cell-autonomous RNAi machinery. *Drosophila* is likewise recalcitrant to environmental RNA, but larvae fed with dsRNAs formulated with transfection agents exhibited both specific target gene knockdown and significant mortality (Whyard et al., 2009), suggesting that the presence of a robust systemic RNAi response may not be a prerequisite for RNAi-mediated insect control.

Extensive RNAi studies have likewise been conducted with dipteran vectors of human diseases to study gene function related to vector biology and vector–pathogen interactions (reviewed in Barnard et al., 2012; Belles, 2010; Manzano-Roman et al., 2012). Although the majority of studies with mosquito species have relied on microinjection of dsRNAs into the hemolymph, other methods such as topical delivery of dsRNA to adults (Pridgeon et al., 2008) and deployment of a recombinant densovirus-mediated RNAi system (Gu et al., 2011) have been described. Several studies have reported success in the oral delivery of dsRNAs to dipteran species, including the mosquitoes, *Aedes aegypti* and *Anopheles gambiae*, and the tsetse fly, *Glossina morsitans morsitans* (Table 5.1). Ingestion of a 10% sucrose solution containing dsRNA (1000 ppm) targeting the vacuolar ATPase subunit A gene resulted in significant knockdown of the target transcript in *A. aegypti* adults as early as 12 h after the onset of feeding, but no mortality data were reported (Coy et al., 2012). Silencing of a P-glycoprotein (P-gp; ATP-dependent efflux pump) gene in *A. aegypti* larvae following ingestion of dsRNA resulted in enhanced sensitivity to the insecticide temephos (Figueira-Mansur et al., 2013), consistent with evidence that P-gp proteins are involved in resistance to organophosphate insecticides. Singh et al. (2013) evaluated several dsRNAs in feeding assays with *A. aegypti* and observed both silencing and mortality/stunting of larvae treated with the β-*tubulin* and *chitin synthase-1* dsRNAs at dietary concentrations of 200–500 ppm. DsRNAs targeting *chitin synthase-2* induced silencing but had no apparent effect on larval survival while silencing of *heat shock protein*

83 manifested itself by increased larval mortality under heat stress. In these studies with mosquito larvae, it is not clear whether the route of RNA delivery is oral or via penetration of the larval cuticle. DsRNA complexed with chitosan and packaged in a gel-based diet was used to silence chitin synthase genes in *A. gambiae* larvae resulting in increased larval sensitivity to agents that either inhibit chitin biosynthesis in insects (diflubenzuron) or act to disrupt the organization or integrity of the peritrophic matrix (calcofluor white, dithiothreitol) (Zhang et al., 2010). Expression of dsRNAs in transgenic *Chlamydomonas* has been proposed as an alternative delivery vehicle for RNAi-mediated control of mosquito larvae (Kumar et al., 2013).

3.3. Lepidoptera

Terenius et al. (2011) provided a useful overview of the status of RNAi studies in lepidopteran species and noted that, with respect to oral delivery, gene suppression only appears to be successful when high concentrations of dsRNA are provided in the diet. In the first reported example, Turner et al. (2006) achieved a significant suppression of several target genes in the brown apple moth, *Epiphyas postvittana* (Walker), by droplet feeding a 4000 ppm dsRNA solution. Subsequently, silencing via oral delivery of dsRNA was reported in a wide range of lepidopteran species including the tobacco hornworm, *Manduca sexta*, diamondback moth (DBM), *Plutella xylostella*, beet armyworm, *Spodoptera exigua*, fall armyworm, *Spodoptera frugiperda*, European corn borer, *Ostrinia nubilalis*, sugarcane stem borer, *Chilo infuscatellus*, and the cotton bollworm, *H. armigera* (Bautista et al., 2009; Gong et al., 2011; Khajuria et al., 2010; Mao et al., 2007; Rodríguez-Cabrera et al., 2010; Surakasi et al., 2011; Tian et al., 2009; Whyard et al., 2009; Xiong et al., 2013; Zhang et al., 2012c; Zhu et al., 2012).

For many early RNAi studies, the object was not to kill an insect but to selectively down-regulate a gene to study its function in a metabolic or developmental process (Belles, 2010). For example, environmental RNAi was used to examine the role of two chitinase genes in regulating chitin content in the peritrophic matrix of the European corn borer (Khajuria et al., 2010), to demonstrate that the cytochrome P450 gene, *CYPBG1*, in DBM is involved in larval resistance to the insecticide permethrin (Bautista et al., 2009), to study the role of the beet armyworm β1 subunit integrin (β*Se1*) in development and cellular immunity (Surakasi et al., 2011), and to demonstrate that a serine protease gene in the fall armyworm

plays an important role in the processing of the *B. thuringiensis* Cry1Ca1 insecticidal protein and the insect's pathogen response to Bt toxins (Rodríguez-Cabrera et al., 2010). In these studies, high concentrations of dsRNA (50–2500 ppm) were delivered by droplet feeding, incorporation into artificial diet, or by soaking leaf tissue prior to feeding. Surakasi et al. (2011) reported concentration-dependent mortality upon silencing of the βSe1 subunit gene in the beet armyworm from which one can interpolate an LC_{50} of 100–200 ppm for this topical application. Silencing of the vacuolar ATPase E subunit gene in *M. sexta* resulted in concentration-dependent mortality and an LC_{50} of only 11 ppm (Whyard et al., 2009). Even so, this level of activity is still three orders of magnitude lower than that observed among the sensitive coleopteran species.

Transgenic plants expressing insect-specific dsRNAs have been reported to impact the growth and survival of certain lepidopteran species, most notably the cotton bollworm, *H. armigera*. Larvae of *H. armigera* fed on tobacco plants expressing a dsRNA targeting the *H. armigera* ecdysone receptor (EcR) gene showed elevated mortality (~40% compared to 10% in the *gfp* control group), growth reduction, and significant suppression of the *EcR* transcript (Zhu et al., 2012). Larvae of another lepidopteran species, the beet armyworm *S. exigua*, also showed elevated mortality and development aberrations when fed on the same transgenic plant material, presumably because the *EcR* target sequences in these two species share regions of >21 bp sequence identity (Zhu et al., 2012). Likewise, tobacco plants expressing a dsRNA targeting a moult-regulating transcription factor, HaHR3, were shown to suppress the *HaHR3* transcript in fed *H. armigera* larvae. In this study, larval mortality of 22–30% and >50% mass reduction was observed after 5 days of feeding on transgenic leaf discs (Xiong et al., 2013). Mao et al. (2007) was the first to use RNAi as a means to alter an insect's ability to cope with xenobiotic compounds, in this case gossypol. Transgenic cotton plants expressing a dsRNA hairpin derived from the *H. armigera* gossypol-inducible cytochrome P450 gene *CYP6AE14* showed increased tolerance to the cotton bollworm, *H. armigera* (Mao et al., 2011) but were not lethal to the larvae. This response can be enhanced by co-delivery of a cysteine proteinase to damage the larval peritrophic matrix, leading to higher gossypol accumulation in the midgut and a modest increase in larval stunting when fed on cotton (Mao et al., 2013). This strategy of targeting detoxification mechanisms in the insect midgut to mitigate plant feeding damage seems promising, particularly since it may not require a systemic RNAi response in the insect. In principle, this strategy is no different

than the use of RNAi to restore sensitivity to insecticides among resistant insect species (Bautista et al., 2009; Figueira-Mansur et al., 2013; Tang et al., 2012).

Difficulty in delivering sufficient dsRNA to lepidopteran gut epithelial cells may be inferred from the paper of Gong et al. (2011) which describes the use of chemically modified siRNAs to target the Rieske iron-sulphur protein gene in the DBM. Both 2′-O-methoxy nucleotides and deoxythymidine were incorporated into the sense and antisense strands to stabilize the siRNAs. When provided to the larvae on cabbage leaves, again at relatively high concentrations (6 μg/cm^2), several siRNAs caused suppression of the target gene transcript and mortality. This approach was extended to include 5′ PEG modification of siRNAs designed to target the acetyl cholinesterase (AchE) receptor in the DBM (Gong et al., 2013). One such siRNA, Si-ace2_001, exhibited an LC_{50} of 53.7 ppm when sprayed on DBM-infested cabbage leaf discs. Kumar et al. (2009) also employed modified siRNAs targeting the AchE receptor of *H. armigera*, in this case substituting a pair of deoxythymidine residues at the 3′ end of both the sense- and antisense strands. Larvae showed reduced growth (stunting) and increased pupal malformation when fed diet containing siRNA at ~0.35 ppm. These results suggest that there might not be a strict dsRNA size dependency to the environmental RNAi response in lepidopterans, at least with chemically modified siRNAs. Larger dsRNAs expressed and encapsulated in *Escherichia coli* have also been reported to impact the growth and survival of *S. exigua* and *H. armigera* larvae (Tian et al., 2009; Xiong et al., 2013; Zhang et al., 2013c; Zhu et al., 2012) but neither the concentration of dsRNA in diet nor the effect of dsRNA alone is reported in these studies. Yang and Han (2014) reported that *E. coli*-encapsulated dsRNAs appear to be more effective than naked dsRNA (1000 ppm in diet) in blocking pupation and causing mortality of *H. armigera* larvae. Suppression of a juvenile hormone esterase-related gene in the corn stalk borer, *Sesamia nonagriodes*, via bacterial delivery of dsRNA did not result in a phenotype (Kontogiannatos et al., 2013). In the case of the sugarcane stem borer, *C. infuscatellus*, naked dsRNA (at 250 ppm) and bacterial-expressed dsRNA applied to corn kernels as a diet appeared equally effective in promoting silencing and stunting larval growth (Zhang et al., 2012c).

In some cases, the insect gut may be bypassed by the use of topical sprays that rely on penetration or adsorption through the insect cuticle (Wang et al., 2011). Topical application of dsRNAs (at 50 ppm) targeting larval stage-specific transcripts in the Asian corn borer, *Ostrinia furnacalis*, led to

significant larval mortality and gene silencing at 5 days post-sprays. The route of dsRNA penetration into the larvae is not known but could involve transit to the hemolymph via the tracheoles. Many of the treatments in this study caused significant mortality in the absence of significant gene silencing at day 3 (Wang et al., 2011), suggesting that either a non-RNAi mechanism is at work or the method used to measure transcript knockdown (qRT-PCR on whole insects) could not detect localized tissue-specific silencing leading to mortality.

3.4. Hemiptera

RNAi techniques have been used successfully in a wide variety of hemipteran species encompassing phloem feeders such as aphids and piercing–sucking insects such as plant bugs (Li et al., 2013b). Since many hemipteran species are relatively small and fragile as nymphs, oral delivery of dsRNAs for gene silencing has been an attractive alternative to microinjection. As was the case with lepidopterans, early studies were not necessarily focused on killing insects but on studying gene function (e.g. Belles, 2010; Paim et al., 2013). Across all studies with hemipterans, the dietary concentrations of dsRNA required for silencing and/or lethal phenotypes vary widely, even between studies with the same organism, but tend to be at least three orders of magnitude higher than effective concentrations used with sensitive coleopteran species. Examples of gene silencing following ingestion of dsRNA include the *nitrophorin 2* (*Np2*) gene in the triatomine bug, *Rhodnius prolixus* (Araujo et al., 2006), the *aquaporin 1* (*ApAQP1*) gene in the pea aphid, *Acyrthosiphon pisum* (Shakesby et al., 2009), the vacuolar ATPase subunit E gene in *A. pisum* (Whyard et al., 2009), the trehalose phosphate synthase (*tps*) gene in brown planthopper, *Nilaparvata lugens* (Chen et al., 2010), the gap gene *hunchback* in *A. pisum* (Mao and Zeng, 2012), and the vacuolar ATPase subunit E gene in *N. lugens* (Li et al., 2011a). Drawing from a list of efficacious gene targets identified for the WCR, Upadhyay et al. (2011) reported silencing of the vacuolar ATPase subunit A—and ribosomal protein L9 genes in the whitefly, *Bemisia tabaci*, as well as mortality with LC_{50} values in the 3–11 ppm range. Focusing on gene targets that are highly or specifically expressed in the midgut, Wuriyanghan et al. (2011) demonstrated target gene suppression and lethality in the potato–tomato psyllid, *Bactericerca cockerelli*, when dsRNAs were presented at high concentrations (500–1000 ppm) in a 15% sucrose diet. Both studies also reported lethality upon ingestion of siRNAs, an effect that has not been reported in

coleopteran species. Ingestion of dsRNA (750 ppm) targeting the *hunchback* gene led to target gene suppression and increased mortality of *A. pisum* nymphs fed on an artificial diet (Mao and Zeng, 2012). Tobacco plants expressing a dsRNA targeting the orthologous *hunchback* gene of the peach aphid, *Myzus persicae*, caused ~30% target mRNA suppression and ~13% inhibition of aphid reproduction (Mao and Zeng, 2014). Feeding large dsRNAs for multiple gene targets at dietary concentrations of 7.5 ppm resulted in target gene suppression and increased mortality in the grain aphid, *Sitobion avenae* F. (Zhang et al., 2013b). In this study, feeding 3–7.5 ppm dsRNA of *C002*, a gene encoding an unknown protein required for normal plant foraging behaviour in *A. pisum* (Mutti et al., 2008), was also reported to cause increased mortality, although it is not clear from the literature whether silencing of this gene should even impact feeding on artificial diet (Mutti et al., 2008). In contrast, *Inhibitor of Apoptosis* (*IAP*) dsRNA presented in diet at 1000 ppm had no effect on the survival of tarnished plant bug, *Lygus lineolaris*, nymphs (Allen and Walker, 2012) despite evidence that silencing of *IAP* via dsRNA injection results in increased *Lygus* mortality (Walker and Allen, 2011). Consistent with many studies, dsRNA injection was far more effective than dsRNA feeding in silencing target genes and causing a lethal phenotype in the corn planthopper, *Peregrinus maidis* (Yao et al., 2013). In this study, it is worth noting that early (day 2) and prolonged (day 6) suppression of the vacuolar ATPase subunit B transcript by diet feeding (500 ppm) did not result in the expected lethal phenotype observed via injection while late silencing (day 6) of the vacuolar ATPase subunit D transcript resulted in a detectable lethal phenotype, but at a time point when the majority (~60%) of the control insects had already died. This illustrates the difficulties encountered with artificial diet feeding assays conducted with many hemipteran species: phenotypes caused by environmental RNAi can be slow to emerge and keeping insects alive on an artificial diet for >7 days can be challenging.

A number of research groups apparently eschewed artificial diet feeding assays altogether and evaluated insect feeding on transgenic plants expressing dsRNAs designed to target essential insect genes. Pitino et al. (2011) selected two target genes in the aphid, *M. persicae*, for knockdown using transgenic *Arabidopsis thaliana*; receptor of activated kinase C (*Rack-1*) and *C002*. When fed on transgenic lines expressing the target dsRNAs, *M. persicae* nymphs exhibited a >50% knockdown in target gene expression after 16 days, but only a small reduction in progeny when compared to nymphs fed on the dsGFP control line or on *Rack-1* lines that did not suppress the *Rack-1* gene. Injection of *C002* dsRNA into *A. pisum* nymphs results in both rapid

gene silencing and 100% mortality after 7 days of feeding on plants (Mutti et al., 2006), suggesting that the delivery of dsRNA via plant expression was simply inadequate for control of this species. A similar outcome was reported using transgenic rice plants expressing dsRNAs targeting three midgut-expressed genes in *N. lugens* (Zha et al., 2011). In this study, significant reductions in target transcript levels (as high as 73% for the *Nltry* gene) were detected 2–4 days after feeding but no lethal phenotype was observed.

Comparisons across related studies reveal marked variation in the hemipteran response to environmental RNAi. For example, Chen et al. (2010) reported significant silencing of the trehalose phosphate synthase (*tps*) gene in *N. lugens* at dsRNA diet concentrations of 500 ppm but no silencing at concentrations of <100 ppm while Li et al. (2011a) reported significant silencing of the vacuolar ATPase subunit E gene in *N. lugens* at dsRNA diet concentrations of only 50 ppm. Silencing of the same essential gene in two distantly related hemipteran species can apparently yield different phenotypes: a dsRNA silencing the vacuolar ATPase subunit E gene in *A. pisum* yielded a reported LC_{50} of 3.4 ppm (Whyard et al., 2009) while a dsRNA silencing the *N. lugens* ortholog caused no phenotype (Li et al., 2011a). Moreover, different groups working with the same insect species and gene target have reported conflicting outcomes. Whereas Whyard et al. (2009) reported oral efficacy with a dsRNA targeting the vacuolar ATPase subunit E gene in *A. pisum* and Mutti et al. (2006) reported lethality following injection of *C002* dsRNA in *A. pisum*, Christiaens et al. (2014) failed to observe a phenotypic response with either dsRNA and delivery method in this species. In this case, the decision to deploy a long *COO2* dsRNA rather than pre-diced siRNAs could have influenced the outcome of the injection experiment. Other factors that could account for these disparate results include insect colony source and health, viral load, life stage, dsRNA stability in different diets, and target gene propensity for silencing.

3.5. Other agricultural pests

Environmental RNAi has been studied in a number of other arthropod pests for which systemic RNAi has already been demonstrated via injections studies. Feeding studies with the invasive fire ant, *Solenopsis invicta*, have demonstrated that dsRNAs ingested by worker ants can be brought back to the colony and shared with the brood (trophallaxis), leading to significant mortality in the brood (larval) population. Worker ant feeding on a 10% sucrose diet containing 1000 ppm dsRNA targeting the PBAN/pyrokinin gene resulted in increased mortality among the nursing fourth instar larvae

(Vander Meer and Choi, 2013). In contrast, a dsRNA targeting a guanine nucleotide-binding protein (GNBP) was reported to be directly toxic to *S. invicta* worker ants when presented in diet at 200 ppm (Zhao and Chen, 2013). Although not an insect, spider mites can be a significant agricultural pest in both vegetable- and row crops. Using a dsRNA permeated leaf disc assay, Kwon et al. (2013) demonstrated gene silencing and significant mortality in the two-spotted spider mite, *Tetranychus urticae*, by targeting several genes at applied concentrations of 160 ppm dsRNA. Most of the efficacious targets surveyed, including genes encoding the COPI coatomer β′ subunit, V-ATPAse subunits A and B, and ribosomal protein S4, had been previously identified as efficacious targets in the coleopteran species *D. virgifera* virgifera (Baum et al., 2007; Khajuria et al., 2013) and *L. decemlineata* (Baum et al., 2007; Zhu et al., 2011). The mite, *Varroa destructor*, an ectoparasite of the honey bee, *Apis mellifera*, and a major suspect contributing to the phenomenon known as bee colony collapse disorder, is also sensitive to environmental RNAi but the route of delivery is unusual in this case. Immersion of *Varroa* mites in a saline solution containing dsRNA can lead to gene silencing (Campbell et al., 2010), but so can feeding dsRNA to the honey bees upon which the mites subsequently feed (Garbian et al., 2012). This unique interspecies transfer of dsRNA from bee to mite presents a strategy for control of this debilitating agricultural pest. Finally, it is worth noting here that locust species (order Orthoptera) display a highly sensitive systemic RNAi response but are refractory to environmental RNAi. As little as 15 pg of dsRNA per mg body mass (~10 ng/insect) is required for significant knockdown of target gene expression in the desert locust, *Schistocerca gregaria* (Wynant et al., 2012). As is the case for *T. castaneum*, the systemic response is dose-dependent, continues to increase over time, and can lead to mortality 7 days post-injection. Likewise, the migratory locust, *Locusta migratoria* was observed to be highly responsive to injected dsRNAs, exhibiting a dose-dependent response leading to target gene suppression and lethality, but was not responsive to environmental RNAi (Luo et al., 2013). A survey of the *Locusta* genome revealed putative orthologs for almost all of the genes implicated in dsRNA uptake in *Drosophila melanogaster* (Saleh et al., 2006), another species unresponsive to environmental RNAi.

4. BARRIERS TO DELIVERY AND EFFICACY IN RECALCITRANT SPECIES

Many recent studies have focused on the optimization of RNAi efficiency in recalcitrant species via selection of optimal target genes (Li et al.,

2013a; Wang et al., 2011; Yao et al., 2013; Zhang et al., 2013b). Contrary to this view, the results of dsRNA screens with sensitive coleopteran species yielding high hit rates among "housekeeping" gene targets using low dietary concentrations of dsRNA support the argument that biological barriers to RNAi, not target gene selection, are the problem. Likewise, the need to provide dsRNAs targeting obviously essential genes (e.g. the V-ATPase subunit genes) at dietary concentrations more than 1000-fold higher than are required for an RNAi phenotype in sensitive coleopteran species suggests that there are great inefficiencies in triggering and sustaining an RNAi response in recalcitrant species. It seems unlikely that the selection of alternative gene targets will close this efficacy gap. The absence of RNAi-induced lethal phenotypes in recalcitrant species may simply reflect the degree to which gene silencing actually occurs. While many studies report target gene silencing upon ingestion of dsRNA, the level of transcript knockdown seldom exceeds 60% and silencing is frequently transient (Huvenne and Smagghe, 2010; Li et al., 2013b). In the case of sensitive coleopteran species, target gene knockdown is frequently $\geq$90% (Baum et al., 2007; Bolognesi et al., 2012; Rangasamy and Siegfried, 2012; Zhu et al., 2011). The typical twofold reduction in mRNA transcript levels observed in many species may fall well within the range of normal variation observed in biological systems and, in any case, may not manifest immediately during the course of a feeding assay since the target protein itself needs to turnover first.

If we set aside the question of target gene selection and dsRNA design, the barriers to efficient RNAi in recalcitrant insects can be pictured as discreet steps in the mechanism of action of RNAi. DsRNAs, whether applied topically to an insect diet or expressed in transgenic plants, must be able to survive long enough to be taken up by insect cells. The insect midgut with its brush border membrane topography (Chapter 1) represents a large surface area that facilitates the absorption of nutrients and is the presumed site of entry for dsRNAs in most studies. It is not clear to what extent the peritrophic matrix in lepidopteran- and coleopteran midguts (Hegedus et al., 2009; Lehane, 1997) or the perimicrovillar membrane in hemipteran midguts (Silva et al., 2004) represents a physical barrier to dsRNA delivery. Upon safe arrival at the gut membrane surface, dsRNAs must be efficiently taken up by epithelial cells and delivered to the intracellular RNAi machinery. If the route of cellular uptake involves endocytosis, then release or escape from the endosome becomes a critical step for delivery to the cytoplasm (Varkouhi et al., 2011). The core RNAi machinery, being agnostic with respect to the source of the dsRNA, processes the dsRNA to generate siRNA–RISC complexes that can trigger a silencing event, provided

transcripts of the gene target are present in that host cell. It is likely that expression of the RNAi core machinery, including Dicer and Argonaute proteins, varies among insect cell types and species, contributing to differences in the efficiency of cell-autonomous RNAi. Factors impacting RNA amplification and/or systemic movement of the silencing signal from the site of cellular entry represent additional barriers to RNAi efficiency and the generation of lethal phenotypes.

The alkaline nature of the lepidopteran midgut (Dow, 1992) could certainly represent one barrier to RNA delivery. Across insect orders, the presence of midgut and salivary nucleases that can rapidly degrade dsRNA molecules likely constitutes a more significant barrier (Arimatsu et al., 2007a,b; Christiaens et al., 2014; Furusawa et al., 1993; Liu et al., 2013; Luo et al., 2013; Rodríguez-Cabrera et al., 2010; Terenius et al., 2011; Wynant et al., 2014). In the case of hemipteran species such as the tarnished plant bug, *L. lineolaris*, which engage in extra-oral digestion of plant material prior to ingestion, the presence of dsRNAses in salivary secretions represents a significant barrier to dsRNA survival (Allen and Walker, 2012). Likewise, Christiaens et al. (2014) provided evidence for dsRNA degradation by dsRNAses in both pea aphid salivary secretions and haemolymph. No less than four different midgut-expressed dsRNAses were observed in the desert locust, *S. gregaria* (Wynant et al., 2014), a species that is recalcitrant to ingested dsRNA but exhibits a potent systemic RNAi response to injected dsRNA (Wynant et al., 2012). Knockdown experiments implicated at least one of these nucleases in dsRNA degradation in the midgut. Likewise, Luo et al. (2013) reported the presence of potent dsRNAses in midgut fluid from the migratory locust, *L. migratoria*, and concluded that rapid degradation of dsRNA in the midgut explains the recalcitrant nature of this species to dietary dsRNA. Given the sensitivity of both locust species to injected dsRNAs, it would be worth understanding the extent to which dsRNAses are also expressed in the haemolymph. Finally, the importance of a gut environment hostile to dsRNA is supported by our own observation that nucleases found in gut extracts from *S. frugiperda*, a lepidopteran species, are far more active in degrading dsRNAs than are nucleases found in corn rootworm gut extracts (Fig. 5.1), particularly at an alkaline pH more typical of the lepidopteran midgut.

Inefficient uptake by midgut epithelial cells represents the next barrier to efficacious RNAi-mediated insect control. The mechanism of dsRNA uptake by insect gut epithelial cells has not been defined, even for highly sensitive species such as the WCR. In the nematode, *C. elegans*, several genes

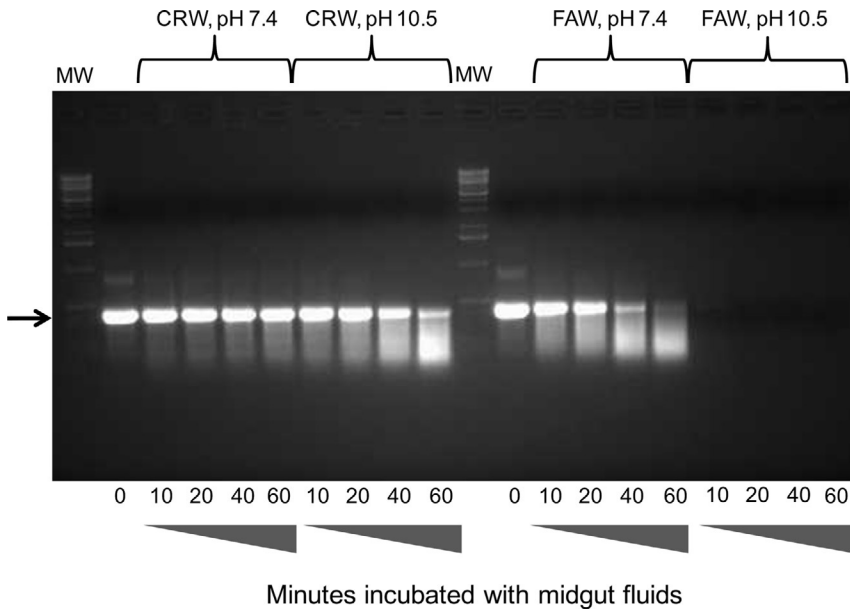

Figure 5.1 dsRNAs are rapidly degraded when treated with midgut extracts from the lepidopteran species, *Spodoptera frugiperda*, but remain intact when treated with midgut extracts from the western corn rootworm, *Diabrotica virgifera* virgifera. A 400-bp V-ATPase A dsRNA (10 μg, see arrow) was incubated at 23 °C with 50 μg of total protein extracted from isolated midgut tissue. Incubations were carried out in 100 μL volumes in either 25 mM Tris HCl (pH 7.4) or 25 mM sodium carbonate (pH 10.5). Aliquots (20 μL) were drawn at the times indicated, the digestions were quenched by ethanol precipitation, and the recovered dsRNAs were resolved by agarose gel electrophoresis. MW = Invitrogen molecular weight standards.

required for environmental RNAi and systemic spread have been identified. The *Sid-1* gene is required for systemic movement of the silencing signal between cells and is also required for environmental RNAi by gut epithelial cells. The *Sid-1* gene encodes a transmembrane protein that acts as a dsRNA-selective and dsRNA-gated channel to passively traffic dsRNAs between cells (Feinberg and Hunter, 2003; Jose et al., 2009; Shih and Hunter, 2011; Shih et al., 2009; Winston et al., 2002). The *Sid-2* gene encodes a membrane protein exclusively localized to intestinal cells that is required for dsRNA uptake from the intestinal lumen (McEwan et al., 2012; Winston et al., 2007). Environmental RNAi therefore requires coordination between the SID-1 and SID-2 proteins. A model has been proposed whereby dsRNAs are imported from the intestinal lumen by SID-2 via endocytosis and released from the internalized vesicles via passive

movement through the SID-1 channel (McEwan et al., 2012; Whangbo and Hunter, 2008). While putative *Sid-1*-like genes have been identified in some insects, there are no reports of *Sid-2*-like genes in insect species whose genomes have been completely sequenced (e.g. Tomoyasu et al., 2008; Xu and Han, 2008; Zha et al., 2011).

In *Drosophila*, an insect lacking *Sid-1*-like genes, evidence has been presented for an energy-dependent endocytic mechanism of dsRNA uptake by cultured (S2) cells, apparently mediated by pattern-recognition scavenger receptors (Saleh et al., 2006; Ulvila et al., 2006). A genomic screen identified 23 genes required for RNAi in S2 cells including many components of the endocytic pathway involved in vesicle trafficking and protein transport or sorting. Subsequently, orthologs of these genes were selected for RNAi screens in *C. elegans*, resulting in the identification of 10 genes whose silencing disrupted the RNAi response triggered by feeding (Saleh et al., 2006). As noted by Hinas et al. (2012), this screen could not distinguish among knockouts that affected dsRNA uptake by intestinal cells, systemic RNAi, or the autonomous cellular RNAi machinery. In a subsequent study, mutations in several of the endocytic pathway genes required for cellular dsRNA uptake in *Drosophila* rendered flies that were hypersensitive to viral infection and unable to mount a systemic antiviral response (Saleh et al., 2009). Further support for the role of endocytic pathways in systemic RNAi proceeded from the characterization of *sid-3* and *sid-5* mutants in *C. elegans*, mutants that are defective in systemic RNAi but are otherwise RNAi competent. *Sid-3* encodes a conserved tyrosine kinase, homologs of which are known to associate with endocytic vesicles, and is required for the import of dsRNA into cells (Jose et al., 2012). *Sid-5* encodes an endosome-associated protein required for the export of dsRNAs from cells (Hinas et al., 2012). Three other mutants identified in RNAi screens, *rsd-2*, *rsd-3*, and *rsd-6*, are insensitive to ingested dsRNA targeting germline-expressed genes but remain sensitive to injected dsRNA (Tijsterman et al., 2004). Other mutants identified in this screen, *rsd-8* and *rsd-4*, were subsequently shown to be alleles of *sid-1* and *sid-2*, respectively, the former already identified as required for systemic RNAi. While the details are not yet understood, we expect that systemic RNAi in insects will prove to be equally complex and that *Sid-1*-like genes will not prove to be the sole determinant of systemic RNAi. Indeed, it is not entirely clear that the *Sid-1*-like genes identified to date in insects are even involved in systemic RNAi. A phylogenetic analysis of Sid-1-like proteins in *Tribolium* suggests that these proteins may not be orthologous to Sid-1, but rather to the *C. elegans* Tag-130 protein that is not involved in

systemic RNAi (Tomoyasu et al., 2008). More recently, Xu et al. (2013) provided evidence through injection studies that the single *Sid-1*-like gene found in *N. lugens* is required for systemic RNAi.

With respect to environmental RNAi, we observe that immortalized insect cells scarcely resemble the differentiated cell types found in an insect midgut. The mechanism of dsRNA uptake by *Drosophila* S2 cells (Saleh et al., 2006; Ulvila et al., 2006) may or may not inform us regarding the mechanism of RNA uptake by gut epithelial cells, particularly since *Drosophila* is not sensitive to ingested dsRNAs in the absence of a transfection agent (Whyard et al, 2009). An alternative endocytic uptake mechanism may be operational in insect species that respond to bacterial-encapsulated dsRNAs in diet (Tian et al., 2009; Xiong et al., 2013; Zhang et al., 2013c; Zhu et al., 2012). In the future, the selection of insects resistant to orally toxic dsRNAs could provide an experimental system to identify genes involved in dsRNA uptake. While *Tribolium* may be the best model for understanding systemic silencing in insects, *Diabrotica* (corn rootworm) or *L. decemlineata* (CPB) may be the emerging model systems for understanding the mechanism of efficient environmental RNAi.

The presence of dsRNAses in the insect hemolymph, noted in some lepidopteran species, could prove to be a formidable barrier to systemic RNAi. A comparative study of the RNAi-sensitive cockroach, *Blattella germanica*, and the RNAi-recalcitrant lepidopteran, *M. sexta*, revealed that dsRNAs are rapidly degraded in the haemolymph fluids from *M. sexta* but not in the haemolymph fluids of *B. germanica* (Garbutt, 2011; Garbutt et al., 2013). Putative orthologs of the *Bombyx mori* and *M. sexta* dsRNAses were also identified in *Spodoptera littoralis* and *S. frugiperda*, two other lepidopteran species insensitive to environmental RNAi. Although not comprising an extensive dataset, these findings nevertheless prompted the suggestion that the capacity to rapidly degrade dsRNA in the hemolymph could represent an evolutionary adaptation to high viral exposure since lepidopteran-specific viruses are abundant and lepidopterans appear particularly prone to viral diseases among holometabolic species (Garbutt, 2011). More recently, Swevers et al. (2013) expanded on this hypothesis to discuss the possible role of persistent virus infections on RNAi efficiency, noting that viruses could interfere with RNAi in insects via number of strategies including expression of RNAi suppressor genes, production of RNA decoys, and manipulation of host gene expression. One attractive feature of this hypothesis is that it might explain why different research groups report such disparate results working with lepidopteran species (Terenius et al., 2011).

5. COMMERCIAL DEVELOPMENT OF RNAi ACTIVES

5.1. Next-generation rootworm-resistant corn

The exceptional sensitivity of corn rootworm larvae to environmental RNAi and the economic importance of this pest complex for corn production in the United States provided an excellent opportunity to develop a biotechnology-based strategy for corn rootworm management. Replicated field trials with transgenic corn plants expressing a corn rootworm V-ATPase dsRNA showed significant root protection (Fig. 5.2) similar to the pot assay results reported by Baum et al (2007).

While these plants showed significant reduction in root damage due to corn rootworm feeding, the damage observed was still above the commercially acceptable threshold of 0.25 for root damage ratings. Genetic crosses between the V-ATPase dsRNA-expressing transgenic event and a corn line expressing the corn rootworm-active Bt protein Cry3Bb1 served to combine these traits. Corn hybrids expressing both traits gave better root protection than did hybrids expressing either Cry3Bb1 or dsRNA alone (Fig. 5.2). The benefit of the RNAi-based trait became even more apparent when adult emergence was assessed. Corn plants expressing the V-ATPase dsRNA

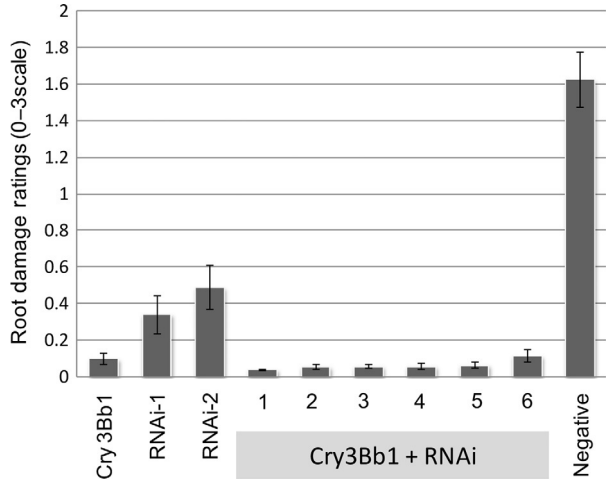

Figure 5.2 Transgenic plants expressing both the Cry3Bb1 protein and corn rootworm (CRW)-specific dsRNAs show excellent protection from rootworm feeding damage. Root damage ratings (0–3 Iowa scale) are averaged across nine locations from four states (IA, IL, IN, NE). Standard error bars are shown.

exhibited superior suppression of adult corn rootworm emergence in growth chamber assays (Fig. 5.3).

The identification of numerous efficacious targets through insect feeding assays (Baum et al., 2007) enabled a rational approach to the disruption of different cellular pathways including, in the case of the *Snf*7 ortholog (Bolognesi et al., 2012), pathways involved in protein trafficking. Ramaseshadri et al. (2013) used histology and immuno-staining to show that knockdown of *Snf*7 gene expression in corn rootworm larvae leads to cellular perturbations that are consistent with a gene encoding an essential component of the ESCORT (Endosomal Sorting Complex Required for Transport) pathway essential for protein trafficking within cells. Further electron microscopy studies (Koči et al., 2014) with WCR midguts have confirmed that loss of *Snf*7 function causes a progressive degeneration of midgut enterocytes resulting in cell sloughing and lysis. These authors conclude that the severe cellular defects caused by loss of Snf7 in midgut enterocytes is the cause of death for rootworm larvae fed *Snf*7 dsRNA. This RNAi-mediated disruption of cellular function represents a very different cause of insect mortality than that attributed to Bt insecticidal proteins, most of which bind to specific midgut receptors and create ion channels and pores to disrupt midgut function in susceptible species (Vachon et al., 2012; Chapter 2). Accordingly, a single transgenic corn line expressing both a corn rootworm-active Bt protein and an efficacious dsRNA (e.g. the *Snf*7 dsRNA) can possess two very different modes of action for the control of

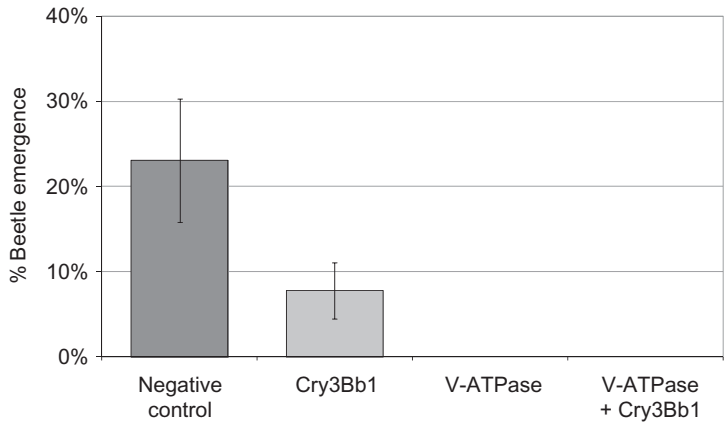

Figure 5.3 Transgenic plants expressing the CRW V-ATPase A dsRNA exhibit superior control of CRW adult beetle emergence in growth chamber assays. Standard error bars are shown.

corn rootworm. Commercial introduction of the *Snf7* RNAi trait in corn hybrids is expected by the end of this decade (Kupferschmidt, 2013).

5.2. Topical application

Given the recalcitrant nature of most lepidopteran and hemipteran pests towards environmental RNAi, the future of RNAi-based insect pest management strategies may depend on the development of topical formulations that facilitate delivery into insect cells. Significant progress has been made in the development of RNA delivery agents for RNAi human therapeutics (Zhou et al., 2013), a field that has wrestled with the challenges of RNA stability, endocytosis, and endosomal escape to deliver siRNA payloads. Examples of delivery agents being explored in the pharmaceutical arena are illustrated in Fig. 5.4.

These include cationic lipids, cationic dendrimers, cyclodextrin polymers, various forms of polethyleneimine, mesoporous silica, cell-internalizing peptides, and nucleic acid aptamers. Transfection agents have been shown to aid in the delivery of dsRNAs to a recalcitrant insect species,

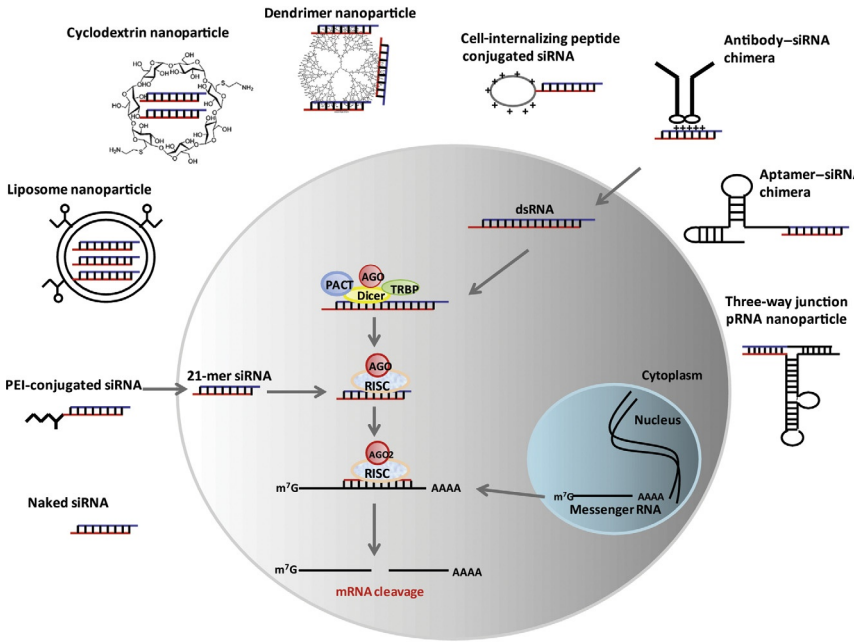

Figure 5.4 The mechanism and delivery strategies for RNA interference. RNAi therapeutics (e.g. siRNA) can be internalized into cells via different delivery vehicles. *Figure reproduced with permission from Zhou et al. (2013).*

in this case *Drosophila* (Whyard et al., 2009). Zhang et al. (2010) demonstrated that chitosan–dsRNA complexes were stabilized in gel-based diets for delivery to mosquito larvae, but the actual impact of chitosan on dsRNA delivery to or uptake by mosquito gut cells was not evaluated in this study. More recently, He et al. (2013) reported that fluorescent nanoparticles (FNPs) comprised of a perylene-3,4,9,10-tetracarboxydiimide chromophore core, a polyphenylene dendrimer inner shell, and a cationic polymer outer shell can serve as a delivery vehicle for both DNA and dsRNA. FNPs coated with a dsRNA targeting the chitinase-like *CHT10* gene were fed at a dietary concentration of 250 ppm to the lepidopteran species, *O. furnacalis*, and shown to be more effective than dsRNA alone in arresting larval development.

A formulation of chemically modified AChE2 siRNA complexed with chitosan was shown to be toxic to DBM larvae in leaf disc assays and in a field trial (Gong et al., 2013). Larval mortality (%) in the field appeared to peak at 5 days post-treatment with the siRNA formulation (50, 100, and 200 ppm treatments), perhaps reflecting foliar degradation of the siRNA active or alternatively an increase in larval infestation leading to a higher recovery of survivors. The high sensitivity of CPB larvae to ingested dsRNAs provides an opportunity to explore the development of dsRNA formulations for use as foliar sprays in insect control. Zhu et al. (2011) evaluated the use of *E. coli*-expressed dsRNA to control CPB, demonstrating consistent target gene knockdown and high mortality of CPB larvae when fed potato leaves treated with either bacterial-expressed dsRNA or *in vitro*-synthesized dsRNA.

The cost of goods weighs heavily on the development of RNAi spray formulations, including the cost of dsRNA production as well as any requisite delivery agents. The first outdoor trial with an RNAi product was conducted by Hunter et al. (2010) to assess the efficacy of a dsRNA product (Remebee-IAPV) in reducing Israeli acute paralytic disease in honey bees, one of many viral diseases that afflict honey bee colonies worldwide. In this case, dsRNA was provided to honey bees in a sugar solution with the aim of blocking virus replication and transmission. Formulations that improve RNA delivery and insecticidal activity while enhancing environmental stability could enable a more favourable cost of goods (through lower use rates) and the development of economically viable topical applications for insect control. Factors that likely impact the environmental stability of dsRNA are not so different from those impacting the stability of synthetic insecticides and include UV damage from exposure to sunlight, microbial degradation

(e.g. on foliar surfaces), and exposure to plant exudates. It is uncertain how formulations that improve the environmental stability of dsRNAs will affect bioavailability and efficacy. Likewise, it is too early in the development of formulation technologies to determine whether approaches taken in the pharmaceutical field can be applied to the development of effective crop protection products.

6. SAFETY CONSIDERATIONS

Safety assessments for the use of RNAi strategies in insect pest management include both the evaluation of environmental safety and food and feed safety. The impact of dsRNAs on non-target organisms, including beneficial insects, and the persistence of applied dsRNAs in the environment, are also addressed during the registration of Bt-based bioinsecticides and insect-protected crops. The current safety assessment approach to biotechnology-derived plants is appropriate for assessing the safety of plants produced using RNA-mediated gene regulation (Codex, 2003; EFSA, 2006; FDA, 1994; Parrott et al., 2010; Petrick et al., 2013; Redenbaugh et al., 1992), and the current environmental risk assessment approach can be applied to characterize risk for RNA-based technologies (Auer and Frederick, 2009; ILSI-CERA, 2011).

RNAi technology has the potential for high specificity towards target pest organisms, even more so than Bt-based technology (Bachman et al., 2013; Baum et al., 2007; Whyard et al., 2009). Larval feeding assays with orthologous CPB- and WCR dsRNAs demonstrated that the accumulation of base pair mismatches between the dsRNA sequence and target gene sequence results in reduced insecticidal activity (Baum et al., 2007). For example, a V-ATPase A dsRNA from WCR sharing 83% sequence identity with the CPB V-ATPase A coding region showed >10-fold lower activity against CPB larvae than did the orthologous CPB V-ATPase A dsRNA. Note that this residual activity towards CPB was expected because the WCR dsRNA sequence still retained stretches of >21 bp sequence identity with the CPB V-ATPase coding region, a result that is entirely consistent with the work of Bolognesi et al. (2012) and Bachman et al. (2013). The case for sequence specificity is further supported by the work of Whyard et al. (2009), who demonstrated that four *Drosophila* species could be selectively controlled using dsRNAs that target the divergent 3′ untranslated region of the γ*Tub23C* tubulin gene, a region lacking 21 bp matches among the four species. Other factors, including the barriers to RNAi described in

this review, clearly play a role in determining sensitivity to dsRNA and assessing risk to non-target organisms. The aggregate of these factors provides outcomes such as those reported by Bachman et al. (2013) showing that a *Snf*7 dsRNA from WCR has no effect when tested on a wide range of insect species. "Indirect" WCR feeding assays with *Snf*7 orthologs from other coleopteran species further demonstrated that the spectrum of activity for the WCR *Snf*7 dsRNA is narrow and only evident in a subset of beetles within the Galerucinae subfamily of Chrysomelidae (>90% identity with the WCR *Snf*7 240 bp dsRNA) that contain a shared sequence length of ≥21 bp within the *Snf*7 coding sequence (Bachman et al., 2013). In summary, the careful selection of dsRNA sequences with no 21 bp match to orthologous genes further reduces the risk of unintended effects on non-target species that may be sensitive to ingested dsRNAs. With respect to RNAi-based strategies for corn rootworm control, however, any assessment of risk towards non-target insects or arachnids should start with the acknowledgement that non-coleopteran species will likely be at least three orders of magnitude less sensitive to environmental RNAi than the target pest itself (Table 5.1).

Regarding food safety, the natural occurrence of long dsRNAs and small RNAs in plants and other foods provides a very long history of safe use by humans (Heisel et al., 2008; Ivashuta et al., 2009; Jensen et al., 2013; Parrott et al., 2010; Petrick et al., 2013), even though sequence complementarity exists between small- and long dsRNAs in crops and human genes (Ivashuta et al., 2009; Jensen et al., 2013). The long list of biological barriers to oral activity of dietary small RNAs and longer dsRNAs in mammals and other vertebrates has been summarized (Petrick et al., 2013). Vertebrate digestive systems display common structural and functional features (Stevens and Hume, 2004); therefore, the same digestive barriers that greatly limit the potential for oral activity of ingested RNA in mammals are likely to be present in lower vertebrates. These barriers include salivary RNAses, harsh acidic conditions in the stomach that lead to depurination and denaturation of nucleic acids, nucleases in the lumen of the gastrointestinal tract, lytic enzymes from pancreatic secretions in the duodenum and nucleases in the blood (Houck and Berman, 1958; Loretz et al., 2006; O'Neill et al., 2011; Park et al., 2006; Petrick et al., 2013). Cellular membranes of the gut epithelium provide a physical barrier to uptake of high molecular weight, hydrophilic compounds like siRNAs (Akhtar, 2009; Jain, 2008). Recent studies employing small RNA sequencing and/or quantitative PCR support the view that dietary dsRNAs have extremely low

bioavailability in mammals, at a level that is orders of magnitude below that needed for biological activity (Cottrill and Chan, 2014; Dickinson et al., 2013; Snow et al., 2013; Witwer and Hirschi, 2014; Witwer et al., 2013). Likewise, a recently reported survey of numerous animal small RNA datasets from public sources has not revealed evidence for any major plant-derived miRNA accumulation in animal samples (Zhang et al., 2012b), consistent with the lack of success in oral delivery of RNA-based therapeutics experienced by the pharmaceutical industry (Petrick et al., 2013). Current pharma approaches employ local and systemic delivery of RNA therapeutics through methods that bypass the oral route (Zhou et al., 2013). Based on the history of safe consumption of dsRNA in the diet, the extensive barriers to ingested RNA, and the lack of any appreciable dsRNA uptake from the diet, dietary dsRNA is not anticipated to represent any hazard or risk to humans, mammals, or other vertebrates.

7. INSECT RESISTANCE MANAGEMENT

In order to reduce the intensity of selection for resistance, insecticides can be applied judiciously when established economic thresholds of pest infestation have been crossed, tank mixed with insecticides providing a different MOA, or rotated with those insecticides during the growing season to alter the selective pressure on insect populations. The strategy of tank-mixing insecticides is fundamentally the same as stacking or pyramiding insect control traits with different MOAs in plants. RNAi-based insecticides would fit well with IPM strategies that rely on the use of multiple control strategies, including synthetic and biological insecticides, and that leverage host plant resistance traits and natural predators to provide economic control of insect pests. The selectivity and slow knockdown anticipated for RNAi insecticidal actives might encourage their use in the context of an IPM framework, perhaps as early-season applications to suppress target pest populations while allowing beneficial insects and predators to thrive.

For transgenic delivery of RNAi-based insect control traits, the path towards resistance management is well worn. Strategies were modelled and deployed in the United States and elsewhere to delay the emergence of insect resistance to insect-protected crops expressing Bt insecticidal proteins (Gassmann et al., 2009; Huang et al., 2011; Tabashnik, 2008). The high dose/refuge strategy for insect-protected crops relies on the out-crossing of rare resistant individuals with sensitive individuals in the non-transgenic refuge, but is only effective if the resistance phenotype is recessive and the dose

sufficiently high to kill heterozygous progeny. In recent years, the stacking of insect resistance traits deploying different MOAs (Roush, 1998) to delay resistance development has become the standard for new commercial transgenic plant offerings. Examples include BollGard® II cotton expressing the Cry1Ac and Cry2Ab proteins for lepidopteran control, Genuity® VT Double PRO® corn expressing the Cry1A.105 and Cry2Ab proteins for lepidopteran control, and Genuity SmartStax® corn expressing the Cry3Bb1 and Cry34Ab–Cry35Ab proteins for corn rootworm control, and the Cry1A.105, Cry1Fa, and Cry2Ab proteins for broad-spectrum lepidopteran control (www.monsanto.com).

To ensure their durability, RNAi-based insect resistance traits should also be stacked with traits employing a different MOA. This is where the work in *C. elegans* provides additional value: the isolation of multiple mutants impaired in environmental and/or systemic RNAi in the nematode provides a cautionary tale for those of us in the field of insect pest management. The risk is not that target genes will be selected for single nucleotide polymorphism variants that evade RNAi processing (large dsRNAs can presumably mitigate this risk; Bachman et al., 2013; Bolognesi et al., 2012) but that, for instance, up-regulation of nucleases or defects in dsRNA uptake, processing, or systemic spread will compromise the triggering and spread of the RNAi response in the pest organism. The observation that most species of *Caenorhabditis* are not sensitive to environmental RNAi (Nuez and Félix, 2012; Whangbo and Hunter, 2008) and that several grassland nematode species are not sensitive to RNAi altogether (Wheeler et al., 2012) further suggests that this capability is under rapid evolution in nematodes. In the case of next-generation rootworm-resistant corn, the stacking of the *Snf7* RNAi trait with Bt genes encoding two rootworm insecticidal toxins with distinct MOAs could provide strong protection for all three traits. Likewise, one can envision the stacking of RNAi traits for lepidopteran control with any number of Bt genes encoding highly efficacious lepidopteran-active proteins. In contrast, the deployment of RNAi traits for hemipteran control is problematic because there are no viable biotech traits that confer effective protection from hemipteran feeding damage and consequently no traits to stack with. Progress has been made in the development of engineered Bt proteins for control of Lygus species in cotton (Baum et al., 2013) but, in general, Bt proteins tend to be ineffective against hemipteran pests. Transgenic plants expressing lectins have been shown to impact the growth, development, and fecundity of hemipteran pests, but regulatory approval of such traits is not assured given their apparent MOA in binding complex

glycans that are also found along the intestinal tract of mammalian herbivores (Vandenborre et al., 2009). Without complementary traits for resistance management, the commercialization of durable RNAi-based hemipteran control traits may prove elusive.

8. CONCLUDING REMARKS

The mechanism of gene suppression manifested as RNAi provides an MOA unique among insecticidal agents. In addition, the potential for highly tailored insecticidal specificity afforded in part by the mechanism of RNAi differentiates it from other insect pest management strategies. Successful development of RNAi actives, either expressed in plants or applied topically as "biological" insecticide sprays, would provide growers with an important tool for sustainable insect pest management.

Despite the exceptional sensitivity of corn rootworm larvae to ingested *V-ATPase*- or *Snf7* dsRNAs, transgenic corn plants expressing these dsRNAs do not provide complete protection from rootworm feeding damage in the field, presumably because of the slow speed-to-kill typical of dsRNA actives. Transgenic corn plants combining the *Snf7* RNAi trait with the Cry3Bb1 gene, however, do provide superior control of corn rootworm larvae and emerging adults in the field. Accordingly, stacking the *Snf7* RNAi trait with suitable Bt traits in corn offers the best opportunity for efficacious and durable control of corn rootworm species. Looking towards the future, it seems likely that the slow speed-to-kill exhibited by insecticidal dsRNAs will affect how these agents are deployed commercially.

It is now firmly established that insect species differ greatly in their response to environmental RNA. Most notably, the lepidopteran and hemipteran species studied to date are far less sensitive to ingested dsRNAs than are the responsive coleopteran species exemplified by the corn rootworm. This differential sensitivity to environmental RNAi helps inform the environmental safety assessment of RNAi for management of coleopteran pests. It will also dictate the approach taken to exploit the phenomenon of RNAi for insect pest management. In order to achieve parity with the WCR *Snf7* dsRNA active, for example, dsRNAs for lepidopteran- or hemipteran pests will need at least a 1000-fold improvement in oral activity. Given the recalcitrant nature of most lepidopteran and hemipteran species to environmental RNAi and the minimal impact target gene selection will likely have on closing this efficacy gap, a clear path towards development of RNAi-based plant traits for lepidopteran- and hemipteran pest

management is not yet apparent. For example, while transgenic plants expressing dsRNAs have been shown to impact the growth and survival of a few lepidopteran or hemipteran species in controlled environment tests, the effects are largely sub-lethal and have not been confirmed with field efficacy data demonstrating economic control of the pest species. Given this apparent "ineffective dose" activity towards recalcitrant species, it would be difficult to justify trait commercialization in light of the significant development and regulatory costs involved (McDougall, 2011; Prado et al., 2014). In the case of hemipteran species, the general lack of complementary (e.g. Bt) traits for use in insect resistance management strategies provides an additional hurdle towards commercial development of transgenic crops employing an RNAi-based trait.

For these reasons, the development of topically applied formulations that facilitate dsRNA delivery into insect cells provides a sensible alternative to the transgenic plant approach and may enable RNAi-based insect pest management for otherwise recalcitrant species. The success of this strategy depends on a detailed understanding of the barriers to efficient environmental RNAi, the development of cost-effective, stable formulations that overcome those barriers, and efficient robust systems for dsRNA production. In some insect species, the absence of an efficient systemic RNAi mechanism may mute any efficiency gained through the use of RNA delivery agents that enable environmental RNAi. Despite these uncertainties, initial studies with formulated dsRNAs suggest this is a promising approach.

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